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Evolution of Marsupial Frogs (Hylidae: Hemiphractinae): Immunological Evidence

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Marsupial tree frogs (Hylidae: Hemiphractinae) share the unusual feature of females brooding eggs on the dorsum or in a dorsal pouch. Results of studies of albumin evolution in these frogs suggest that the generic lineages are ancient (Cretaceous), that a minimum of four species groups can be identified within the genus *Gastrotheca* and that the monotypic *Amphignathodon* should be placed in *Gastrotheca*. The results also support recent suggestions that direct development is the plesiomorphic reproductive mode in egg-brooding hylid frogs. The estimated times of divergence of various lineages coincide with major orogenic events in South America.

Los sapos marsupiales (Hylidae: Hemiphractinae) se caracterizan por transportar las hembras los huevos en el dorso o en un saco dorsal. Los resultados del análisis de la evolución de las albuminas sugieren una divergencia cretácica para dicho género. El género monotípico *Amphignathodon* quizás debería ser relocalizado con el género *Gastrotheca*, dentro del cual se reconocen cuatro grupos de especies. Este arreglo estaría de acuerdo con recientes indicaciones por las cuales el desarrollo directo es un modo reproductivo plesiomórphico en los hylidos marsupiales. Las épocas estimadas de divergencia evolutiva coinciden con los grandes eventos orogénicos en Suramérica.

STUDIES of albumin evolution have provided evidence of relationships among taxa in several anuran families over the past decade (Maxson et al., 1982; Maxson, 1984; Maxson and Roberts, 1985). Because of their unusual mode of reproduction, the approx. 60 species of egg-brooding hylid frogs of the subfamily Hemiphractinae (including Amphignathodontinae) have been the subject of several evolutionary studies in recent years (Duellman and Hoogmoed, 1984; Wassersug and Duellman, 1984; Duellman and Hillis, 1987). Frogs in this subfamily share a unique embryonic feature—large, membranous, external gills that completely (or partially) envelop the embryos, all of which develop on the dorsum of females (*Cryptobatrachus, Hemiphractus,* and *Stefania*) or in a brood pouch on the dorsum of females (*Amphignathodon, Flectonotus, Fritziana,* and *Gastrotheca*). In *Amphignathodon, Cryptobatrachus, Hemiphractus, Stefania,* and most species of *Gastrotheca,* the eggs hatch directly into froglets; there is no free-living tadpole stage. In *Flectonotus* and *Fritziana,* the eggs hatch into advanced, nonfeeding tadpoles that complete their development in a few days in water in bromeliads or tree holes (Duellman and Gray, 1983). The free-living, feeding tadpoles produced by some species of *Gastrotheca* have a lengthy development period in ponds.

There seems to be a simple orthogenetic progression in the reduction of larval features of the oral morphology in *Gastrotheca*; thus, free-living tadpoles have the most completely developed mouthparts, whereas embryos of direct-developing species of *Gastrotheca* and other genera of hemiphractines have reduced mouthparts (Wassersug and Duellman, 1984). These authors suggested that the oral morphology of direct-developing species of *Gastrotheca* can be accounted for by simple truncation of the normal tadpole developmental program. Thus, the

tadpole-producing species of *Gastrotheca* may have evolved from lineages having direct development. A preliminary immunological study of *Gastrotheca* (Scanlan et al., 1980) showed that direct development existed in several lineages of *Gastrotheca*; thus, either direct development or the free-living larval condition may have arisen independently several times in the genus.

A cladistic analysis of allozymes of the high-Andean groups of *Gastrotheca* (Duellman and Hillis, 1987) supported the recognition of northern (*G. plumbea*) and southern (*G. marsupiata*) groups of high-Andean *Gastrotheca,* as suggested on the basis of immunological evidence by Scanlan et al. (1980). Furthermore, the allozymic analysis showed that plesiomorphic alleles were shared by the two montane groups and the lowland species having direct development. This reproductive mode occurs in most of the species in the *G. marsupiata* group and in two members of the *G. plumbea* group. However, allozymic data suggest that tadpole production is plesiomorphic in the *G. plumbea* group and that direct development in *G. orophylax* and *G. plumbea* is derived. Thus, the question remains: In the egg-brooding hylid frogs, is direct development primitive or derived? In order to test these alternatives, we have expanded our earlier immunological study (Scanlan et al., 1980) to include all genera of hemiphractine frogs and many additional species of *Gastrotheca.* In so doing, we also have examined the immunological relationships among the taxa in light of their taxonomy and biogeography.

Materials and Methods

Albumin antisera were prepared according to established procedures (Maxson et al., 1979). For species represented by a small sample, only one or two (rather than the standard three) rabbits were immunized to elicit antisera. The number of rabbits used for antisera production is indicated parenthetically in the following list. Comparative studies of antisera from single vs multiple rabbits indicate little variation among rabbits in estimates of albumin immunological distances (Maxson, 1981; Busack et al., 1988). In addition to the antisera (to *G. excubitor, G. marsupiata, G. ochoai, G. orophylax,* and *G. riobambae*) used by Scanlan et al. (1980), the following antisera were prepared: *Cryptobatrachus fuhrmanni* (2), *Flectonotus pygmaeus* (1), *Gastrotheca argenteovirens* (1), *G. dendronastes* (3), *G. (Amphignathodon) guentheri* (3), *G. longipes* (3), *G. monticola* (1), *G. nicefori* (1) and *G. pseustes* (1). Additionally, plasma or muscle tissue preserved in phenoxyethanol (Maxson and Wilson, 1975) were used as albumin samples from 18 populations of *G. pseustes,* seven populations of *G. riobambae,* 17 additional species of *Gastrotheca, Flectonotus fitzgeraldi, Fritziana goeldii, Hemiphractus bubalus, H. fasciatus, Stefania evansi* and *S. ginesi* (see Appendix 1 for museum numbers for voucher specimens and locality data).

Several frogs from the same population were pooled in order to obtain sufficient plasma for producing antisera. Otherwise, all albumins used in micro-complement fixation (MC'F) tests were maintained as individual samples. Several individuals from randomly selected populations of *G. riobambae* and *G. pseustes* were tested initially to ascertain levels of intrapopulational variation in albumin. In all cases, immunological distances (ID) from different individuals varied by only 1 or 2 units, the same amount of variation reported in other studies (Maxson, 1981; Maxson and Syzmura, 1984). The samples of *G. riobambae* and *G. pseustes* used as antigens for comparisons in Tables 1, 3–4 were the homologous populations used for producing antisera.

All antisera were used in the quantitative micro-complement fixation (MC'F) assay (Champion et al., 1974; Maxson and Maxson, 1986) to estimate albumin evolution in these frogs. Data are reported as immunological distances (ID). For albumin it has been determined that one unit of ID is approximately equivalent to one amino acid difference between the albumins compared (Maxson and Wilson, 1975; Maxson and Maxson, 1986) and that approx. 10 such substitutions accumulate every 5.5–6 million years of lineage independence (Wilson et al., 1977). Divergence times are reported as ranges calculated by multiplying the smallest ID by 0.55 and the largest ID by 0.60 so as to give the broadest time range possible. Sarich (1985) reevaluated the calibration of the albumin molecular clock in some placental mammals (based on quantitative precipitin data, not MC'F), but we choose to use the former calibration because it is based on MC'F data, includes more extensive vertebrate comparisons, and includes data on diverse groups of anurans.

The tree (Fig. 1) is a consensus of two trees, each constructed from averages of reciprocal ID presented in Tables 1 (experimental data) and 2 (adjusted data). Each tree was drawn using a modification of Farris' (1972) Wagner tree

algorithm. Because the variance in the estimated ID increases more rapidly than linearly with increasing ID (Maxson and Maxson, 1986), only the phylogenetically closest lineages are used to calculate limb lengths for the branches on the tree. This contrasts to the methods of Farris (1972) and Fitch and Margoliash (1967) wherein averages of all outside lineages generally are used to calculate limb lengths. Our method provides results that incorporate less "noise" than the use of global optimization procedures. The most distant lineages are used to root the tree. This method has been determined to be appropriate for albumin MC'F data in which the large number (580) of amino acid positions in albumins permits the assumption that there are few parallel and back-mutation contaminations in the data (Maxson, 1984; Maxson and Maxson, 1986).

Results

The average titer for the antisera used in this study is 4400 (range 2000–8000) and the average slope is 390. These values are typical for antisera to anuran albumins (Maxson, 1984). All antisera were judged to be directed predominantly to serum albumin as evidenced by a single precipitin arc in immunoelectrophoresis when tested with whole serum. The results of MC'F tests using purified albumin, whole plasma, or muscle extracts as the antigen source were indistinguishable.

Results of all MC'F tests are presented in Tables 1, 3–4. The percent standard deviation from perfect reciprocity for the nine antisera in Table 1 is 23.7%. When small ID (≤ 9) are involved in calculations, the standard deviation appears to be inflated (Scanlan et al., 1980). The standard deviation drops to 10.5% when the comparisons involving ID of ≤ 9 are omitted. When the correction procedure of Sarich and Cronin (1976) for data exhibiting a significant nonrandom element in the distribution of nonreciprocities is applied (Table 2), the standard deviation is 17.6% for all pairs and 8.5% for those comparisons of ID ≥ 10 (Table 2). Another way of evaluating the reciprocal estimates is by the average deviation in reciprocal ID estimates of all pairs (Scanlan et al., 1980). For the entire data set (Table 1), this is 4.4 units, approximately twice the value expected (Maxson and Maxson, 1979). For the adjusted data set (Table 2), this value is 3.3 units.

Some of the data in Tables 1, 3–4 were reported by Scanlan et al. (1980). In those instances where the ID are not identical to those reported earlier, the new ID are the result of work that has been repeated using new individuals, or the reactions have been attempted at higher ID. For example, we used a different sample of *G. argenteovirens* to prepare antisera than that studied in 1980, and we report an ID of 8 to *G. orophylax*, compared to the ID of 6 reported previously. The major discrepancy is seen in the comparison of *G. riobambae* to *G. nicefori*; Scanlan et al. (1980) reported an ID of 6, whereas herein we report an ID of 17 and an average of 19 ID for the reciprocal comparisons. The *G. nicefori* used in 1980 (KU 181071) was from La Delicia, in the Cordillera Oriental of extreme western Venezuela, whereas those used in the present study (Appendix 1) come from the Cordillera Oriental and Cordillera Central of southern Colombia, some 400 and 700 km, respectively, from the Venezuelan locality. Conceivably these represent immunologically distinct species that have not been distinguished morphologically.

Several values are missing in Tables 1, 3–4. Samples of many species used for heterologous one-way comparisons were represented by only small pieces of muscle tissue. Therefore, once a species had been tested with representative antisera, further tests were not run. At distances over 60 ID, exhaustive comparisons were not run in order to conserve scarce material. More importantly, it has been shown that ID of more than 100 do not provide good phylogenetic information (Maxson and Maxson, 1986). Thus, once it has been ascertained that lineages are more than 100 units apart, it is not necessary to attempt to refine the ID more precisely. Different lower estimates reported from the same antiserum generally are due to variable antigen availability.

The tree calculated by using the averages of the reciprocal data (Table 1) had the same branching pattern as that based on the adjusted reciprocal data (Table 2). The tree (Fig. 1) is a consensus of the two trees, and branching nodes are indicated at average immunological distances. A trichotomy of *G. orophylax*, *G. argenteovirens*, and *G. riobambae* exists because all values measured with the antiserum to *G. riobambae* were quite low. Moreover, the data for this triad (Table 1) do not satisfy the triangle inequality; the lineage leading to *G. riobambae* has a distance of 0 or -1. However, the adjusted data (Table 2) suggest that *G. orophylax* and *G. riobambae* are

TABLE 1. MATRIX OF RECIPROCAL IMMUNOLOGICAL DISTANCES AMONG *Gastrotheca*.

Antigens	Antisera										
	OR	AR	RI	MO	OC	EX	MA	PS	NI	DE	LO
G. orophylax (OR)	0	9	4	11	20	16	11	14	23	100	—*
G. argenteovirens (AR)	8	0	3	20	31	21	20	24	22	—	—
G. riobambae (RI)	1	5	0	12	19	13	9	17	21	107	92
G. monticola (MO)	6	19	8	0	31	20	21	20	23	—	—
G. ochoai (OC)	19	24	12	26	0	3	6	7	38	100	—
G. excubitor (EX)	17	23	10	22	10	0	5	4	30	122	99
G. marsupiata (MA)	18	26	13	27	20	8	0	4	40	121	—
G. pseustes (PS)	16	27	16	26	24	10	4	0	40	—	—
G. nicefori (NI)	21	19	17	23	45	22	32	38	0	117	—
G. dendronastes (DE)	—	—	90	—	—	—	—	—	—	0	96
G. longipes (LO)	—	—	118	—	—	—	—	—	113	94	0

* Reactions not performed because distant placement was confirmed (see Table 3).

sister species. Nonetheless, the distance along the limb leading to these two taxa is less than 2 units; generally, such short lengths are not considered to be significant and are collapsed (Maxson and Wilson, 1975), for such small numbers approach the limits of resolution of the MC'F technique (Maxson and Maxson, 1979).

Typically, trees are evaluated by comparing the experimental data with the distances determined from the reconstructed tree and by calculating either a percent standard deviation (Fitch and Margoliash, 1967) or a percent error (Prager and Wilson, 1978). The data reported herein are unusual in that many species are very close immunologically and have such variable rates of albumin change (or "noise") that construction of a definitive tree is difficult. For example, production of a tree without negative branches does not seem to be possible with these data. This problem indicates that errors in estimating albumin-sequence evolution are relatively large. Accordingly, we present a conservative interpretation of the data wherein some of the possible sublineages are suppressed. The data do support a definitive *G. marsupiata* group, but the lineages within the *G. plumbea* group, especially *G. monticola*, are not so well defined. No standard evaluation of how well the tree fits the data is presented, because such an evaluation would have little meaning for a consensus tree such as we have constructed.

Table 3 shows the results of one-way comparisons representative of the four species groups of *Gastrotheca*. These data allow us to infer the relative positions of groups of all species on a generalized species group phenogram (Fig. 2). We also compared representative species of *Gastrotheca* and other hemiphractines with antisera to three other hemiphractine genera (Table 4).

DISCUSSION

Relationships among hemiphractine genera.—Antisera were available only to four of the seven living genera of hylid marsupial frogs. Results from comparisons among some of these frogs are presented in Table 4. Albumin differentiation among the genera is substantial; many comparisons are beyond the range (>120–150 ID) within which MC'F is able to measure accurately the immunological distances (Maxson and Maxson, 1986). Accordingly, we refrain from making definitive statements concerning branching relationships for these genera beyond noting that they diverged from one another more than 60 million years before present (MYBP).

Morphologically and developmentally, *Flectonotus* and *Fritziana* are sister groups (Duellman and Gray, 1983; Wassersug and Duellman, 1984); the ID of about 100, indicating a separation of 55–60 million years, supports this relationship. Likewise, *Cryptobatrachus* and *Stefania* are one another's closest relatives based on morphological characters (Duellman and Hoogmoed, 1984). This relationship is supported by developmental data (Wassersug and Duellman, 1984). If the high ID values that we obtained between these genera reflect their relative times of divergence, our data suggest that *Cryptobatrachus* is more distant to *Flectonotus* (ID = >175 = 96–105 MYBP) than to *G. rio-*

TABLE 2. MATRIX OF RECIPROCAL IMMUNOLOGICAL DISTANCES AMONG *Gastrotheca*, AFTER APPLYING THE CORRECTION METHOD OF SARICH AND CRONIN (1976).

Antigens	Antisera										
	OR	AR	RI	MO	OC	EX	MA	PS	NI	DE	LO
G. orophylax (OR)	0	9	5	10	14	16	16	17	23	100	—
G. argenteovirens (AR)	8	0	4	18	21	21	29	29	22	—	—
G. riobambae (RI)	1	5	0	11	13	13	13	21	21	107	92
G. monticola (MO)	6	19	9	0	21	20	30	25	23	—	—
G. ochoai ((OC)	19	24	14	23	0	3	9	9	38	100	—
G. excubitor (EX)	17	23	12	19	7	0	7	5	30	122	99
G. marsupiata (MA)	18	26	15	24	14	8	0	5	40	121	—
G. pseustes (PS)	16	27	19	23	16	10	6	0	40	—	—
G. nicefori (NI)	21	19	20	20	30	22	46	47	0	117	—
G. dendronastes (DE)	—	—	99	—	—	—	—	—	—	0	96
G. longipes (LO)	—	—	130	—	—	—	—	—	113	94	0

bambae (mean ID = >146 = 80–88 MYBP), and that the latter is more distant to *Flectonotus* (ID = >180 = 99–108 MYBP) than to the others.

The relatively close ID of 100–104 measured between *G. riobambae* and *Stefania* and the 115 ID between *Flectonotus* and *Stefania* are inconsistent with the large distances measured between *G. riobambae* and *Flectonotus*. However, in many cases, the *Flectonotus* antibody gives lower values than reciprocal estimates. Thus, until we can make an antibody to *Stefania*, we reserve judgement on the precise placement of this lineage among the Hemiphractinae.

The morphologically bizarre *Hemiphractus* with posterolaterally projecting paraoccipital processes, fanglike teeth, and enlarged neural spines of the vertebrate protruding dorsally is distinct from other hemiphractine hylids (Trueb, 1974). However, it shares with *Cryptobatrachus* and *Stefania* the development of eggs into froglets openly on the back of females and in having embryos that lack all features associated with free-living, feeding tadpoles. (*Stefania* has a remnant of an upper beak and apparently a vestigial ventral velum [Wassersug and Duellman, 1984].) Immunologically, *Hemiphractus* seems to be more distant to *Cryptobatrachus* (ID = 157 = 86–94 MYPB) than to *Flectonotus* (ID = 90 = 50–54 MYBP). Note, however, that the *Flectonotus* antibody again is the low estimate.

The monotypic *Amphignathodon* is unique among anurans in having true teeth on the dentary; otherwise, morphologically and developmentally it is like *G. cornuta* and *G. dendronastes* (Duellman, 1983; Wassersug and Duellman, 1984). Immunologically, it is no more distinct from some species of *Gastrotheca* than these are from other congeners (Fig. 2). For example, comparisons of albumins of *G. longipes* and *G. dendronastes* to antibodies of *Amphignathodon guentheri* provide ID of 118 and 115, respectively, whereas the reciprocal values are 101 and 109, respectively. The ID of *G. riobambae* to *G. longipes*, *G. dendronastes*, and *A. guentheri* are 92, 107, and 90, respectively. Thus, we consider *A. guentheri* to be a member of the genus *Gastrotheca*. Accordingly, the generic name *Amphignathodon* Boulenger 1882 becomes a junior synonym of *Gastrotheca* Duméril and Bibron 1841. Henceforth, we refer to this species as *G. guentheri*.

The ID of various species of *Gastrotheca* to the other genera of egg-brooding hylids are highly variable (90 → 193; Table 4). The values of *G. riobambae* and *G. guentheri* to *Cryptobatrachus* are reasonably consistent (ID = >130; ≥163 and >146; 168, respectively). These indicate a divergence time of 72–101 MYBP. Comparisons between various species of *Gastrotheca* and *Flectonotus* range from 90–>180 ID (50–108 MYBP). The values between *Hemiphractus* and species of *Gastrotheca* also are variable (ID = >95–>186), thereby suggesting a divergence time of 52–112 MYBP. As indicated above, the comparisons with *Flectonotus* are inconsistent. Because we did not have additional antisera to more taxa and had very little *Flectonotus* antigen remaining, we could not perform additional comparisons to elucidate this situation.

The immunological data provide only an approximation of the possible relationships of the genera of hemiphractine frogs because the great

TABLE 3. ONE-WAY COMPARISONS OF REPRESENTATIVES OF MAJOR SPECIES GROUPS IN THE GENUS *Gastrotheca*.

Antigens	Antisera											
	AR	MO	OR	RI	EX	MA	OC	PS	NI	DE	LO	GU
G. plumbea group												
G. argenteovirens (AR)	0	20	8	3	21	20	31	24	22	—	—	—
G. aureomaculata	1	—	7	4	17	—	—	—	—	109	—	—
*G. dunni**	14	—	—	5	—	22	—	24	—	—	—	—
*G. litonedis**	—	10	—	2	—	—	—	12	—	—	—	—
G. monticola (MO)	19	0	6	8	20	21	31	20	23	—	—	—
G. orophylax (OR)	9	11	0	4	16	11	20	14	23	100	>100	94
G. plumbea	5	17	3	4	14	—	—	14	—	—	—	—
G. psychrophila	—	—	0	2	—	—	—	11	—	—	—	—
G. riobambae (RI)**	5	12	1	0	13	9	19	17	21	107	92	90
*G. ruizi**	—	11	—	4	—	—	—	19	—	—	—	—
G. trachyceps	5	—	12	8	—	—	—	28	—	—	—	—
G. marsupiata group												
G. christiani	20	—	—	12	17	11	21	—	—	101	—	—
G. chrysosticta	20	—	—	10	—	13	—	12	—	—	—	—
G. excubitor (EX)	23	22	17	10	0	5	10	4	30	122	99	100
G. galeata	19	17	14	12	8	9	21	6	—	—	—	—
G. griswoldi	—	21	—	12	10	8	19	4	—	—	—	—
G. marsupiata (MA)	26	27	18	13	8	0	20	4	40	121	—	100
G. ochoai (OC)	24	26	19	12	3	6	0	7	38	100	>100	100
G. peruana	—	22	13	11	12	7	25	10	—	—	—	—
G. pseustes (PS)*	27	26	16	16	10	4	24	0	40	—	—	—
G. nicefori group												
G. nicefori (NI)	19	23	21	17	22	32	45	38	0	117	>100	>106
G. ovifera group												
G. andaquiensis	—	—	—	101	—	—	—	—	100	84	23	99
G. cornuta	—	—	—	115	—	—	—	—	—	42	—	—
G. dendronastes (DE)	—	—	—	90	—	—	—	—	—	0	96	115
G. guentheri (GU)	—	—	72	86	83	—	—	—	123	109	101	0
*G. helenae****	—	—	(53)	(39)	(51)	—	—	—	—	—	47	—
G. longipes (LO)	—	—	—	118	—	—	—	—	113	94	0	118
*G. ovifera****	—	—	(50)	(53)	(54)	—	—	—	95	45	—	—
*G. weinlandii****	—	—	(40)	(17)	(35)	—	—	—	95	—	27	—

* Formerly included in *G. riobambae*.
** Includes *G. cavia* of Scanlan et al. (1980) synonymized with *G. riobambae* by Duellman and Hillis (1987).
*** ID in parentheses were reported by Scanlan et al. (1980) and could not be repeated because of shortage of antigens; see text for explanation.

distances are at, or beyond, the point of resolution by MC'F techniques (Maxson and Maxson, 1986). Values are indicated as approximate or greater than, when we had insufficient antigen to carry out additional comparisons. In most of these instances, the MC'F curves were broadening and requiring so much antigen that it was apparent we had reached the limits of phylogenetic resolution of the albumin MC'F methodology (Maxson and Maxson, 1986). Therefore, we can say only that the lineages are quite distinct and have been independent for a long time. Most of generic differentiation presumably occurred in the Cretaceous. Resolution of a branching pattern of relationships must await the application of other techniques, such as sequence analysis of ribosomal DNA.

Relationships among the species of Gastrotheca.— For the purposes of this discussion, we recognize four species groups of *Gastrotheca* (Table 3; Fig. 2). The *G. ovifera* group contains large species that have direct development and inhabit tropical lowlands and lower montane for-

ests; species for which immunological data are available include *G. andaquiensis, G. cornuta, G. dendronastes, G. guentheri, G. helenae, G. longipes, G. ovifera,* and *G. weinlandii.* The *G. plumbea* group contains small to moderate-sized species that produce tadpoles or have direct development and inhabit the high Andes north of the Huancabamba Depression in northern Peru; included in this group are (* = direct development): *G. argenteovirens, G. aureomaculata, G. dunni, G. litonedis, G. monticola, G. orophylax,* G. plumbea,* G. psychrophila, G. riobambae, G. ruizi,* and *G. trachyceps.* The *G. marsupiata* group contains small to moderate-sized species that produce tadpoles or have direct development and inhabit the high Andes to the south of the Huancabamba Depression (except *G. pseustes,* which occurs in the Andes of Ecuador); included in this group are (* = direct development): *G. christiani,* G. chrysosticta, G. excubitor,* G. galeata,* G. griswoldi,* G. marsupiata, G. ochoai,* G. peruana,* and *G. pseustes. Gastrotheca nicefori,* a moderately large species that has direct development and inhabits montane forests in northwestern South America, is in its own group.

A preliminary analysis of immunological distances among *Gastrotheca* (Scanlan et al., 1980) suggested that northern Andean species (*G. plumbea* group) are consistent in their distances from a southern Andean species, *G. excubitor* (*G. marsupiata* group) and that both of these groups are more distant from the large, lowland species (*G. ovifera* group). The expanded data set reported here confirms these suggestions and allows the resolution of the immunological relationships of many species of *Gastrotheca.*

Immunologically, there are three subgroups in the *G. ovifera* group (Table 3; Fig. 2). The four species on the eastern slopes of the Andes and in the upper Amazon Basin (*G. andaquiensis, G. helenae, G. longipes,* and *G. weinlandii*) form one subgroup; the ID of three of these species to *G. longipes* are 23–47, indicating divergence times of 13–28 MYBP. *Gastrotheca cornuta* and *G. dendronastes* on the Pacific slopes of the Andes are about 42 ID apart; this suggests a divergence of about 23–25 MYBP. *Gastrotheca ovifera* in the Cordillera de la Costa in Venezuela is about equally differentiated from *G. dendronastes* (ID = 45; 25–27 MYBP). These three species constitute a second subgroup. The third subgroup consists of *G. guentheri*; it is about 110 ID from each of the other subgroups, which have an average of about 95 ID from one another (Table 3). These distances suggest that the three subgroups have been genetically independent from one another for more than 50 million years.

Other ID among members of the *G. ovifera* group (Table 3) were reported by Scanlan et al. (1980). Because of lack of material, these tests could not be rerun after antisera were prepared to other members of the *G. ovifera* group. The earlier work (Scanlan et al., 1980) showed that three species (*G. helenae, G. ovifera,* and *G. weinlandii*) were quite distant from the *G. marsupiata* and *G. plumbea* groups. However, these ID (35–71, $\bar{x} = 49$) are considerably lower than the ID (72–118, $\bar{x} = 95$) of other members of the *G. ovifera* group (*G. andaquiensis, G. cornuta, G. dendronastes, G. guentheri,* and *G. longipes*) to species in the *G. marsupiata* and *G. plumbea* groups. These inconsistencies do not seem to be the result of the experimental work. Therefore, until additional material is available, these discrepancies cannot be resolved.

Within the *G. plumbea* group, Scanlan et al. (1980) reported what appeared to be a cryptic species within the samples identified as *G. riobambae* and noted that some of these samples were immunologically closer to the *G. marsupiata* group than to *G. riobambae* in the *G. plumbea* group. Subsequent analyses of morphology and allozymes by Duellman and Hillis (1987) revealed that *G. "riobambae"* was a composite of several species, including *G. espeletia* (no immunological data available), *G. litonedis, G. riobambae,* and *G. pseustes.* One other closely related species, *G. ruizi,* was recognized by Duellman and Burrowes (1986). With the exception of *G. pseustes,* all of these species belong to one species group, but *G. pseustes* shares 11 derived allozymic electromorphs with members of the *G. marsupiata* group (Duellman and Hillis, 1987).

Using antisera prepared to *G. riobambae, G. pseustes,* and *G. monticola,* we sampled many populations of *Gastrotheca* from the Andes of Ecuador and sorted each according to its albumin cross-reactivity. Because all comparisons involved albumins from individual frogs, we were able to detect frogs that had been misidentified. All frogs had been allocated tentatively to *G. riobambae.* MC'F tests to *G. riobambae* antisera showed 18 individuals with ID of 10–18; these same individuals had an average ID of 25 to antisera of *G. monticola.* Later all of these frogs were confirmed to be *G. pseustes* (and had ID of 0–1 to an antibody made to *G. pseustes*). Individual samples of true *G. riobambae* had an av-

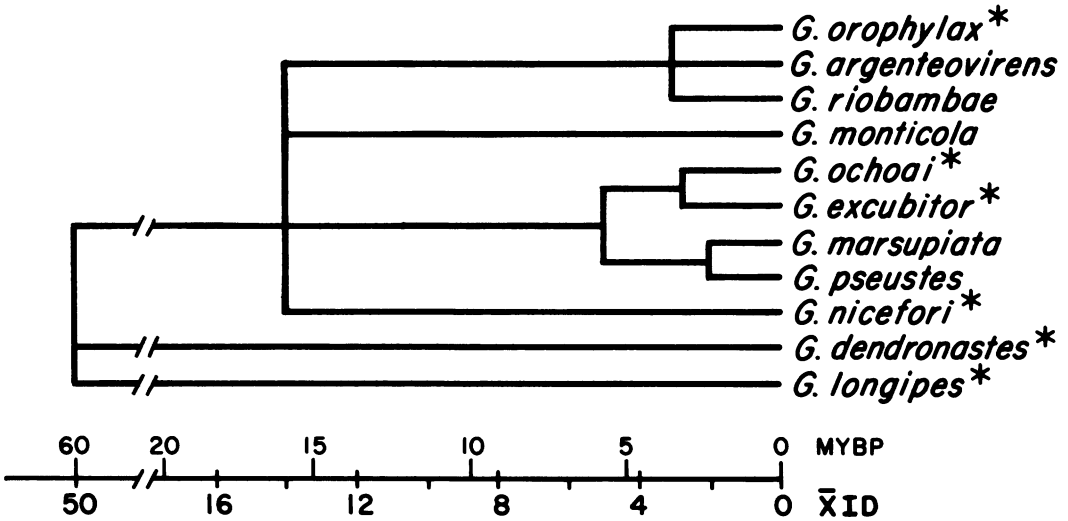

Fig. 1. Immunological relationships of species of *Gastrotheca* based on reciprocal ID. This is a consensus tree based on data in Tables 1–2. The upper scale is in millions of years before the present (MYBP) and the lower is in average ID units. Thus, the scale is in albumin ID per lineage and the values in MYBP are half those indicated in Table 5. Asterisks indicate species having direct development. See text for discussion.

erage of 11 ID to the *G. pseustes* antisera and 9 ID to *G. monticola*. The average distances among these species confirmed the placement of *G. pseustes* in the *G. marsupiata* group (Fig. 1; Duellman and Hillis, 1987). Although Scalan et al. (1980) determined that there were several potential cryptic species included in *G. "riobambae,"* thorough allozymic and morphological studies were required to define the taxa. As noted above, MC'F can identify reproductively isolated populations, but for recent speciations, analysis of multiple loci is needed to make explicit specific identifications (Maxson and Maxson, 1979).

Three species in the *G. plumbea* group in the Andes of southern Colombia have small ID. The morphologically distinct *G. aureomaculata* is immunologically like *G. argenteovirens* (ID = 1), whereas *G. trachyceps* is more distant from *G. argenteovirens* (ID = 5). According to Duellman (1987), *G. argenteovirens, G. trachyceps,* and *G. dunni* are closely related allopatric species; however, the cross-reactions that we have for *G. dunni* show it to be somewhat more distant to *G. argenteovirens* (ID = 14) than to *G. riobambae* (ID = 5). *Gastrotheca trachyceps* seems to be only slightly closer to *G. argenteovirens* (ID = 5) than to *G. riobambae* (ID = 8). These four species may have differentiated from one another 3–10 MYBP.

With the exception of comparisons with the *G. monticola* antisera and antigen, we found small ID among the species of the *G. plumbea* group. The ID are ≤14, indicating that divergence has occurred <8.4 MYBP. Our data are not in conflict with the phylogenetic relationships of these species proposed on the basis of allozymic evolution by Duellman and Hillis (1987).

Immunologically, *G. monticola* is most distant to other members of the *G. plumbea* group. The greatest distance is to *G. argenteovirens* (ID = 19.5 = 11–12 MYBP). The ID to most other members of the *G. plumbea* group are 6–12, indicating divergence from those species 3–7 MYBP. These data conflict somewhat with the phylogeny proposed by Duellman and Hillis (1987), who placed *G. monticola* and *G. litonedis* as sister species sharing five derived electromorphs; these two species share three derived electromorphs with *G. psychrophila,* and together these species form a sister group to the remainder of the Ecuadorian species of the *G. plumbea* group.

The immunological data indicate that the albumin of *G. litonedis* is more similar to that of *G. riobambae* (ID = 2) than to that of *G. monticola*

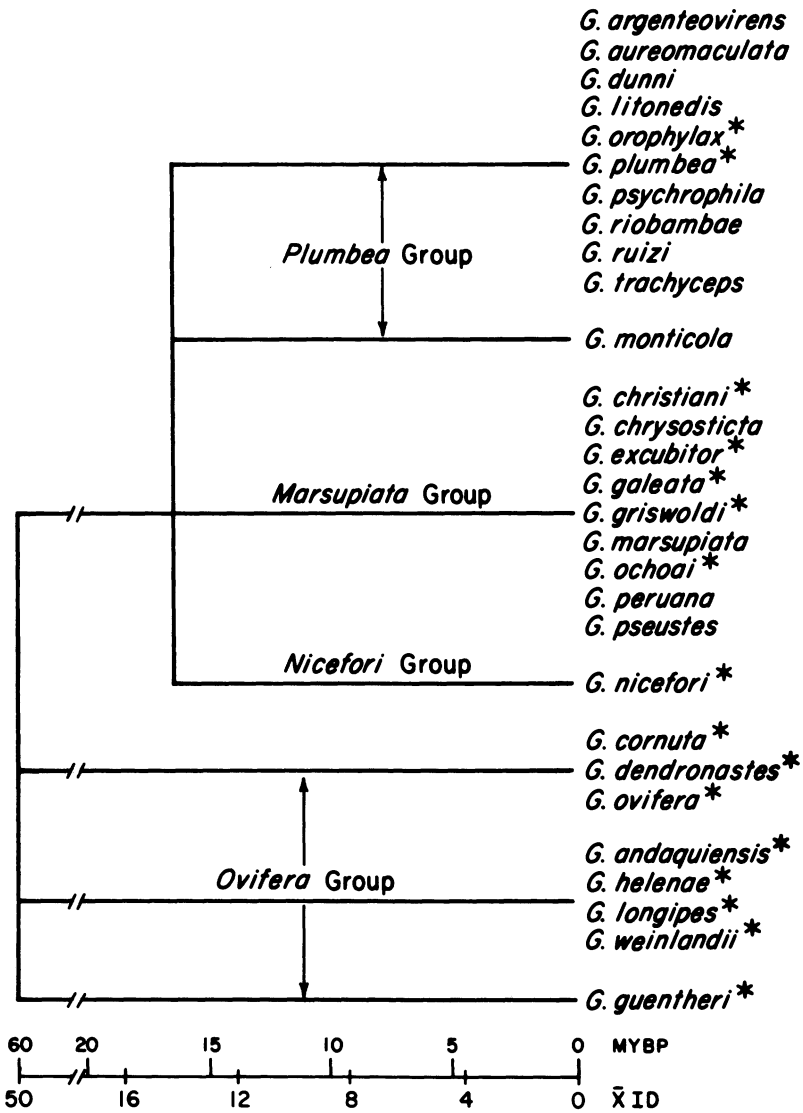

Fig. 2. Immunological relationships of species of *Gastrotheca* based on reciprocal ID data (see Fig. 1) and unidirectional tests in Table 3. Asterisks indicate species having direct development.

(ID = 10). Similarly, *G. psychrophila* is only 2 units from *G. riobambae*. Data for other species in the *G. plumbea* group are concordant with the tree given by Duellman and Hillis (1987). In fact, a correlation analysis performed on our tree (Fig. 1) and that of Duellman and Hillis (1987) gives a correlation of 0.88. As indicated earlier, these immunological data are not sufficiently robust to define sublineages within the *G. plumbea* group, and this accounts for the apparent discrepancies between the interpretations of the electromorphic and immunological data. All of the ID to *G. monticola* are high, but these high values do not necessarily separate *G. monticola* from the *G. plumbea* group. These data provide an excellent example of why it is better to perform electrophoresis of many loci rather than compare albumins for species sharing a very recent common ancestor (Maxson and Maxson, 1979). That *G. monticola* is not a member of the *G. marsupiata* group is attested to by the higher ID (17–27, $\bar{x}$ = 23) of seven species

TABLE 4. ONE-WAY COMPARISONS AMONG TAXA OF HEMIPHRACTINE FROGS.

Antigens	Antisera					
	CR	FL	GU	DE	LO	RI
Cryptobatrachus fuhrmanni (CR)**	0	>175	>146	>170	>193	>130*
Flectonotus pygmaeus (FL)	>175	0	>147	>110	95	>180
F. fitzgeraldi	***	9	—	—	—	—
Fritziana goeldii	—	100	—	—	—	—
Hemiphractus bubalus	157	—	—	147	>95	136
H. fasciatus	—	90	141	—	>186	120
*Stefania evansi***	155	115	>124	164	—	104
S. ginesi	—	—	—	—	—	100
Gastrotheca guentheri (GU)	168	>130	0	109	101	86
G. dendronastes (DE)	—	90	115	0	96	90
G. longipes (LO)	—	95	118	94	0	118
G. riobambae (RI)	>163	>110	90	107	92	0

* This value differs from that reported in Scanlan et al. (1980) and is a better estimate of the distance.
** Reported as *Cryptobatrachus boulengeri* by Scanlan et al. (1980).
*** Missing comparisons were not made because of shortage of antigens.
**** Reported as *Stefania scalae* by Scanlan et al. (1980).

in that group to *G. monticola* and the allozymic evidence presented by Duellman and Hillis (1987).

Some populations referred to *G. monticola* on external morphological characters have high ID to other populations. One subadult specimen (KU 181741) from 20.5 km SSW of Leimebamba, Departamento de Amazonas, Peru, has ID of 15 to *G. monticola* (antibody of population from Pomacochas, Departamento de Amazonas, Peru), and of 4–6 to *G. pseustes*, *G. marsupiata*, and *G. excubitor* (all members of the *G. marsupiata* group), respectively. Specimens (KU 181733–34) from El Tambo on the ridge to the west of Huancabamba, Departamento de Piura, Peru, have an ID of 26 to the sample from Pomacochas, but an ID of only 3 to *G. marsupiata*, a species from which they are distinct morphologically. Therefore, it is likely that at least two unnamed cryptic species exist in the mountains of northern Peru (Duellman and Trueb, 1988).

The ID among species in the *G. marsupiata* group generally are higher (3–25, $\bar{x} = 11$) than those among species in the *G. plumbea* group (excluding *G. monticola*, 0–14, $\bar{x} = 5$). The distances obtained from unidirectional tests of various species to antisera of *G. excubitor*, *G. marsupiata*, *G. ochoai*, and *G. marsupiata* form a reasonably consistent pattern, but some reciprocal values are inconsistent (Tables 1, 3). For example, the reciprocal values of *G. ochoai* and *G. excubitor* are 10 and 3, between *G. ochoai* and *G. marsupiata* 20 and 6, and between *G. ochoai* and *G. pseustes* 24 and 7. As can be seen in all of these comparisons, the *G. ochoai* antibody gives ID higher than the average by about 50%. Thus, the placement of this lineage is considered in light of this strong nonrandom directionality of ID estimates.

Among the Peruvian species in the *G. marsupiata* group, the two tadpole-producing species (*G. marsupiata* and *G. peruana*), together with the Ecuadorian tadpole-producing species (*G. pseustes*), seem to form a natural subgroup (ID = 4–10 = 2.2–6.0 MYBP). Likewise, three species having direct development (*G. excubitor*, *G. griswoldi*, and *G. ochoai*) seem to form another subgroup. The ID betweeen *G. griswoldi* and *G. excubitor* is 10 (5.5–6.0 MYBP), and the average distance between the latter and *G. ochoai* is 6.5 (3.6–3.9 MYBP). However, the ID of *G. griswoldi* to *G. ochoai* is 19 (13 when adjusted, as in Table 2), greater than the IDs to *G. marsupiata* and *G. pseustes*—8 and 4, respectively. The direct-developing *G. galeata* is intermediate between these subgroups; the ID to *G. excubitor* is 8 and to *G. pseustes* and *G. marsupiata* 6 and 9, respectively. Again, the distance to *G. ochoai* is much greater (ID = 21; 14 adjusted by the Sarich-Cronin [1976] method).

The only available data on the two species from northern Argentina (*G. chrysosticta* that produces tadpoles and *G. christiani* that has direct development) are unidirectional tests. These data suggest that *G. christiani* and *G. chrysosticta*

may be intermediate between the *marsupiata* and *plumbea* groups, but no inference about their relationship to one another can be made without an antibody to either taxon.

Morphologically, *G. nicefori* resembles some members of the *G. ovifera* group, but immunologically it is closer to members of the *G. plumbea* and *G. marsupiata* groups (Tables 1 and 3). The averages of reciprocal ID between *G. nicefori* and species in the *G. plumbea* group are 19–23 ($\bar{x}$ = 21), species in the *G. marsupiata* group 26–42 ($\bar{x}$ = 36), and species in the *G. ovifera* group 100–123 ($\bar{x}$ > 112). Thus, *G. nicefori* possibly represents a sister group to the *G. plumbea* and *marsupiata* groups, and all of these are a sister group to the *G. ovifera* group.

Tadpoles vs direct development.—It is generally accepted that reproduction via aquatic eggs and tadpoles is the primitive reproductive mode in anurans, a mode shared with primitive salamanders and caecilians (Duellman and Trueb, 1986). Free-living, feeding larvae are characteristic of three of the four subfamilies of hylids (Hylinae, Pelodryadinae, and Phyllomedusinae) and occur in some species of *Gastrotheca* in the Hemiphractinae, whereas some hemiphractines (*Flectonotus* and *Fritziana*) have free-living nonfeeding tadpoles, and most hemiphractines have direct development—no free-living larval stage. Based solely on these three types of development, it is most parsimonious to consider free-living, feeding tadpoles, as exemplified by some species of *Gastrotheca*, as the primitive reproductive mode in the Hemiphractinae. In this arrangement, free-living, nonfeeding tadpoles and direct development either could be derived sequentially or independently from free-living, feeding tadpoles. Sequential derivation of direct development from free-living, feeding tadpoles is consistent with the oral morphology of embryos and larvae (Wassersug and Duellman, 1984).

If this arrangement is accepted, the high-Andean species of *Gastrotheca* would have to be considered as the outgroup to other hemiphractines, whereas *Cryptobatrachus* and *Hemiphractus* would be the most derived of the groups having direct development. Data on pouch structure and embryonic gills (del Pino, 1980; del Pino and Escobar, 1981; Duellman, 1985), morphology (Duellman and Gray, 1983; Duellman and Hoogmoed, 1984), allozymic evolution in *Gastrotheca* (Duellman and Hillis, 1987), and the present data on immunological distances cast serious doubt on this simple orthogenetic progression.

The high ID among the genera of hemiphractine frogs show unequivocally that these genera have been in existence for a long period of time, at least since the Cretaceous. The only members of the subfamily that have free-living larvae are *Fritziana*, *Flectonotus*, and some high-Andean species of *Gastrotheca*. Morphologically, *Flectonotus* seems to be derived from *Fritziana*, and the *Fritziana-Flectonotus* lineage is distinct from *Gastrotheca* (Duellman and Gray, 1983). This sequence and independent evolution also are apparent in the oral morphology of the embryos and larvae, as shown by Wassersug and Duellman (1984), who emphasized that the orthogenetic progression of free-living tadpoles to direct development was too simplistic in light of morphological and immunological evidence. They concluded that because the oral features of direct-developing *Gastrotheca* were similar to, if not identical with, those at some stage in the ontogeny of free-living larvae, all of the morphological patterns observed in direct-developing *Gastrotheca* can be accounted for by simple truncation of larval development.

If we assume that direct development is the plesiomorphic state in hemiphractine hylids, free-living tadpoles are independently derived in the *Fritziana-Flectonotus* lineage and in *Gastrotheca*. In fact, free-living tadpoles possibly evolved independently in the *G. plumbea* and *G. marsupiata* groups. Based on immunological data and the allozymic data presented by Duellman and Hillis (1987), it is possible to construct three equally parsimonious cladograms to incorporate the changes in reproductive modes in these two groups (Fig. 3). The first alternative assumes a change from direct development to tadpole production in the common lineage to the two groups; this requires independent reversals to direct development in lineages within both groups (Fig. 3A). The other alternatives assume direct development in the common lineage to the two groups. In one of these alternatives, tadpole production is acquired independently in at least one lineage within the *G. marsupiata* group and in at least two lineages within the *G. plumbea* group (Fig. 3B). In the other alternative, tadpole production is acquired independently in at least one lineage in the *G. marsupiata* group and in the lineage to the *G. plumbea* group; this requires a reversal to direct development in one lineage within the *G. plumbea* group (Fig. 3C).

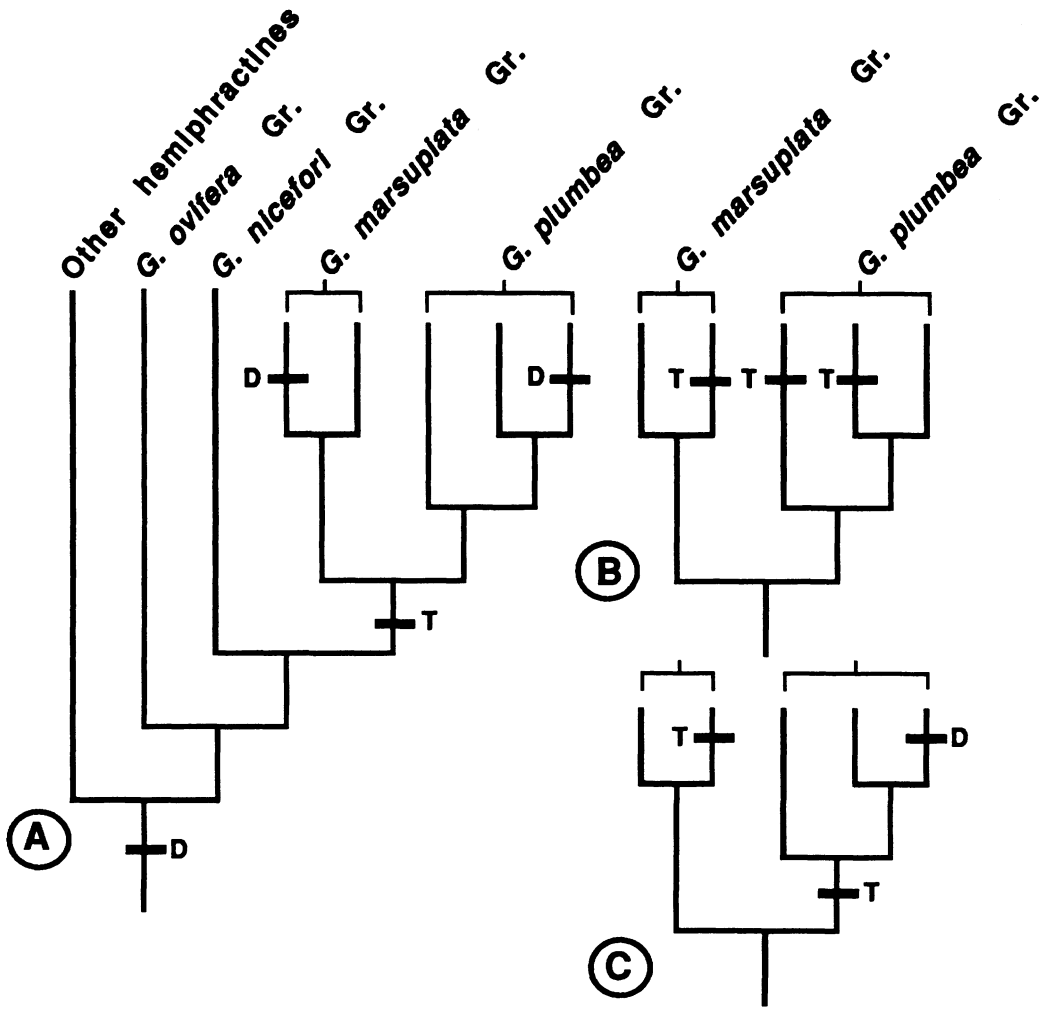

Fig. 3. Alternative phylogenetic hypotheses for the evolution of reproductive mode in the *Gastrotheca marsupiata* and *G. plumbea* groups. Shifts in reproductive mode are indicated by tick marks on lineages; D = change to direct development, T = change to free-living tadpoles.

As noted earlier, the two Argentine species (*G. christiani* and *G. chrysosticta*) may be one another's closest relatives; if so, tadpoles were acquired independently in *G. chrysosticta* from those in the other tadpole-producing lineage in the *G. marsupiata* group. The available data are insufficient to resolve this matter. Thus, although the immunological data have helped to resolve the question of the evolutionary relationships among the hemiphractine frogs in general, available data are not adequate to assess the precise evolutionary sequences in reproductive modes (production of tadpoles or froglets) in the *G. marsupiata* and *G. plumbea* groups in the high Andes.

Biogeography.—Application of the albumin molecular clock (Wilson et al., 1977) with an accumulation of 10 amino acid substitutions each 5.5–6 million years of independent evolution allows the estimation of the ages of lineages and permits the correlation of these predictions with known events in earth history. The biogeographical history of hemiphractine hylids is particularly interesting because of the apparent antiquity of the group and because they inhabit such diverse regions as the ancient Guiana Shield and the geologically recent Andes.

The differentiation of *Flectonotus, Fritziana, Stefania,* and *Hemiphractus* from one another seems to have taken place in the Late Creta-

TABLE 5. ESTIMATED TIMES OF DIVERGENCE OF THE GENERA OF HEMIPHRACTINE FROGS. Values are millions of years before present (MYBP).*

	CR	FL	FR	GA	HE	ST
Cryptobatrachus (CR)	0					
Flectonotus (FL)	96–105	0				
Fritziana (FR)	—	55–60	0			
Gastrotheca (GA)	72–116	50–108	—	0		
Hemiphractus (HE)	86–94	50–54	—	52–112	0	
Stefania (ST)	85–93	63–69	—	55–98	—	0

* See text for calculation of ranges.

ceous or Early Cenozoic (≤70 MYBP). *Gastrotheca* and *Cryptobatrachus* presumably were distinct lineages for a longer time (Table 5). *Stefania* is restricted to the Guiana Shield, and *Fritziana* is endemic to the Brazilian Shield. These two regions have been continuously emergent throughout the Mesozoic, Cenozoic, and Quaternary. Presumably, the shields were continuous with one another until the Late Cretaceous when elevation of both was initiated, and the subsidence of the Amazon Basin began (Beurlen, 1980; Valeton, 1973). There is no geological evidence for a past highland connection between the Guiana Shield and the northern Andes (Haffer, 1974), the region inhabited by *Cryptobatrachus*. Therefore, it is probable that an ancestral hemiphractine existed on the ancient shields and in western tropical South America before the Late Cretaceous. Uplift of the Guiana Shield resulted in isolation of *Stefania* from *Cryptobatrachus*, and the uplift of the Brazilian Shield resulted in the isolation of *Fritziana*, whereas *Flectonotus* was restricted to the Cordillera de la Costa in northern Venezuela; this range was uplifted in the Late Cretaceous through the Oligocene (Liddle, 1946). The estimated times of divergence of the genera are consistent with this scenario.

The table-top mountains (tepuis) of the present Guiana Highlands were formed by erosional dissection of a continuous tableland during the Cenozoic. Species of *Stefania* have allopatric distributions on these tepuis (Duellman and Hoogmoed, 1984). Unfortunately, without an antibody to *Stefania* we are unable to estimate genetic distances between the species of *Stefania*.

Flectonotus fitzgeraldi on the islands of Tobago and Trinidad has an ID of 9 to *F. pygmaeus* in the Cordillera de la Costa in northern Venezuela. Thus, the differentiation of these species took place about 5 MYBP in the Pliocene. Duellman and Gray (1983) allowed that Hardy's (1982) suggestion that *Flectonotus* reached the Caribbean islands by waif dispersal was plausible.

The five species of *Hemiphractus* inhabit the upper Amazon Basin and the lower slopes of the Andes. Immunological distances between *Hemiphractus* and *Flectonotus* indicate a separation in the Early Cenozoic (50–54 MYBP). The differentiation of *Hemiphractus* from the *Flectonotus-Fritziana* lineage may be associated with the subsidence of the Amazon Basin, which occurred approximately 65 MYBP (Beurlen, 1970).

The historical events that resulted in the vicariance of *Gastrotheca* from other hemiphractines are shrouded in uncertainty. Certainly the genus was present in western South America prior to the initial uplift of the Andes in the Late Cretaceous. However, there are two species of the *G. ovifera* group (*G. fissipes* and *G. microdiscus*) in southeastern Brazil (Duellman, 1984). Immunological data are lacking for these species, so it is not possible to determine the times of their differentiation from other members of the genus or other genera of hemiphractine hylids. We cannot rule out the possibility that the early history of *Gastrotheca* was associated with the Brazilian Shield. However, the great diversity of *Gastrotheca* in the Andes and the antiquity of some of the species there suggests that the Brazilian species were derived from Andean lineages that in the past had continuous distributions across the southern part of the Brazilian Shield, which most closely approaches the Andes in southwestern Brazil.

The uplift of the Andes began in the late Cretaceous (>60 MYBP), but until the Miocene few areas were more than 1000 m above sea level. In the Miocene (±20 MYBP), the Andes to the south of the Huancabamba Depression from Peru to northern Argentina were uplifted further (Aubodin et al., 1973); the final major

orogeny of these ranges took place at the end of the Plioene (±2 MYBP), and there was some additional elevation in the Pleistocene (Gansser, 1973; James, 1973). The Andes to the north of the Huancabamba Depression in Ecuador and Colombia were not uplifted more than 1000 m above sea level until the Pliocene; the major orogeny of these ranges took place at the end of the Pliocene (van der Hammen et al., 1973; Shagam, 1975; Simpson, 1979).

Two or three glaciations occurred in the Pleistocene in the northern Andes; during times of maximum glaciation, temperatures were depressed by 6–7 C and environments were shifted downward by 1000–2000 m (Vuilleumier, 1971; van der Hammen, 1974). Pleistocene and Recent glaciations depressed snow lines by as much as 1500 m during the last two glaciations in the Peruvian Andes (Hastenrath, 1967; Simpson, 1979).

These historical events explain the evolutionary history of Andean *Gastrotheca* as evidenced by their immunological relationships. The enigmatic *G. guentheri* presumably diverged on the Pacific lowlands from the rest of the *G. ovifera* group in the Paleocene (56 MYBP). Presumably, another stock of these forest-dwelling *Gastrotheca* crossed the low Andean ranges in the Eocene (>46 MYBP), resulting in the differentiation of the *G. cornuta-dendronastes-ovifera* lineages on the Pacific slopes from the *G. andaquiensis-helenae-longipes-weinlandii* lineage on the Amazonian slopes. During the Miocene and Pliocene, species in both of these lineages differentiated, as evidenced by estimated times of divergence of 23–27 MYBP in the former lineages and 13–28 MYBP in the latter. Equitable climatic conditions and continuous forests across the low Andes were prevalent in the Early Cenozoic, but by the Miocene orogenic events must have resulted in altitudinal differentiation of habitats, which in turn resulted in isolation of populations of these forest-dwelling *Gastrotheca*.

The lineage that gave rise to the *G. nicefori*, *G. plumbea*, and *G. marsupiata* groups apparently differentiated from the lineage giving rise to the *G. ovifera* group in the Early Cenozoic; the lowest estimates of time of differentiation between the groups is 50–54 MYBP. Speciation in the *G. marsupiata* group began about 10 MYBP, at the end of the Miocene when the central Andes were uplifted. Although some speciation occurred in the *G. plumbea* group at the end of the Miocene (10–12 MYBP for *G. monticola* from *G. argenteovirens*) and during the Pliocene (8 MYBP for *G. dunni* from *G. argenteovirens*), most speciation in the group occurred at the end of the Pliocene and early Pleistocene (<3 MYBP).

The timing of speciation among the high-Andean species of *Gastrotheca* coincides with the major Andean orogenies and Pleistocene events. Many of the high-Andean species inhabit páramos, habitats that were nonexistent in the northern Andes until the end of the Pliocene (van der Hammen, 1974). All of the tadpole-producing species of *Gastrotheca* live in these supra-treeline habitats. The recent evolution of these habitats and the recent times of divergence of the tadpole-producing species support the contention that this is a derived reproductive mode in *Gastrotheca*.

Hemiphractus fasciatus, *Gastrotheca cornuta*, and *G. nicefori* occur in forested habitats in northwestern South America and Panama. These are the only hemiphractine hylids in Central America. They must have dispersed into Central America after the closure of the Panamanian Portal in the late Pliocene (3–5 MYBP).

Unresolved problems.—In addition to obtaining more reciprocal cross-reactions so as to refine the resolution of immunological distances among the various taxa that have been studied, data need to be obtained for additional taxa. This is especially critical for species of *Cryptobatrachus*, *Fritziana*, and *Hemiphractus* for which no data are available on the ID among species in those genera.

Two major unresolved problems exist in *Gastrotheca*. One concerns the geographically disjunct species, *G. fissipes* and *G. microdiscus*, in eastern Brazil. These species are far removed geographically from other *Gastrotheca* and are morphologically distinct from one another (Duellman, 1984). The second problem deals with two species, *G. walkeri* and *G. williamsoni*, in northern Venezuela. Superficially, these species resemble *G. longipes* in the upper Amazon Basin. However, the structure of their brood pouch is unique. Instead of a single opening to a dorsal pouch, there is a pair of openings to paired retroperitoneal pouches, in which the embryos undergo direct development. Data on the immunological relationships of these four species to other species of *Gastrotheca* should help to resolve their phylogenetic position within the genus.

Appendix 1

Specimens from which blood and/or muscle were obtained for MC'F. LM numbers refer to Maxson's catalogue of tissues at the University of Illinois; these numbers are followed by the museum acronym and catalogue number(s) for the voucher specimens. Museum acronyms follow Leviton et al. (1985) with the additions that EP = Eugenia del Pino, Quito, Ecuador, and SG = Stefan Gorzula, Caracas, Venezuela.

Cryptobatrachus fuhrmanni.—LM 76, ICNMNH 4756–57, Colombia: Santander: San Gil. *Flectonotus fitzgeraldi.*—LM 491, KU 192399–400, Trinidad: Simla, Arima Valley. *F. pygmaeus.*—LM 77, KU 184958–59, Venezuela: Aragua: Estación Biológica Rancho Grande, 1100 m. *Fritziana goeldii.*—LM 793, USNM 232355, Brazil: Rio de Janeiro, Itatiaia, Brejo da Lapa, 1800–2000 m.

Gastrotheca andaquiensis.—LM 9, KU 179737, Ecuador: Pastaza: 8.1 km NW Mera, 1270 m. *G. argenteovirens.*—LM 348, KU 181168–73, Colombia: Cauca: 2 km E. Silvia, 2550 m. *G. aureomaculata.*—LM 80, KU 181194, Colombia: Cauca: Moscapán, 14.7 km W Leticia, 2050 m. *G. christiani.*—LM 54–55, FML 2881, Argentina: Jujuy: Quebrada Abra de Cañas, Parque Nacional Calilegua, 1700 m. *G. chrysosticta.*—LM 60, FML 2864, Argentina: Jujuy: Finca Arazayal, Depto. Orán. *G. cornuta.*—LM 807, AMNH 107251, Panama: Bocas del Toro: Río Changuinola, near Quebrada El Guapo, 90–170 m. *G. dendronastes.*—LM 1, KU 183830–33, Colombia: Valle: Río Calima, 1.5 km W Lago Calima, 1230 m. *G. dunni.*—LM 83, ICNMNH 10059, Colombia: Antioquia, 3.5 km (by road) N Llanos de Cuiva, 2700 m. *G. excubitor.*—LM 364, KU 173193–94, Peru: Cuzco: Abra Acanacu, 25 km NNE Paucartambo, 3520 m.

Gastrotheca galeata.—LM 370, KU 181700, Peru: Piura: 15 km E Canchaque, 1850 m. *G. griswoldi.*—LM 372, KU 181701–04, Peru: Junín: 6 km ENE Paccha, 3840 m. *G. guentheri.*—LM 75, MCZ 108616, Ecuador: Pichincha: Quebrada La Plata, 2.1 km E Tandapi. *G. helenae.*—LM 379, KU 181070, Venezuela: Táchira: N slope Cerro Tamá, 3250 m. *G. litonedis.*—LM 1089, KU 202690, Ecuador: Azuay: 10 km NW Girón, 2750 m. *G. longipes.*—LM 2, USNM 258905, Peru: Amazonas: Caterpiza. *G. marsupiata.*—LM 3, KU 173202–04, Peru: Cuzco: Río Tancac, 23 km NW Ollantaytambo, 3850 m. *G. monticola.*—LM 351, KU 181747–53, Peru: Amazonas: Pomacochas, 2180 m. *G. nicefori.*—LM 52, ICHMHN 6398, Colombia: Huila: 7 km (by road) NW San José de Isnos, 1950 m; LM 875, ICNMHN 4771, Colombia: Meta: Acacías, road from Guayabetal to Mazanares, 1400 m. *G. ochoai.*—LM 87, KU 173499–500, Peru: Cuzco: Chilca, 10 km N Ollantaytambo, 2760 m. *G. orophylax.*—LM 4, KU 178570, Ecuador: Carchi: 5.7 km NW El Carmelo, 2910 m; LM 2180, KU 178568, Ecuador: Napo: 11 km ESE Papallacta, 2660 m. *G. ovifera.*—LM 378, KU 185785, Venezuela: Aragua: Estación Biológica Rancho Grande, 1100 m. *G. peruana.*—LM 369, KU 181740, Peru: Cajamarca: 23 km SW Celendin, 3050 m. *G. plumbea.*—LM 1168, KU 202698, Ecuador: Cotopaxi: Pilaló, 2320 m.

Gastrotheca pseustes.—LM 1235–37, KU 178565–67, Ecuador: Azuay: Cuenca, 2650 m; LM 1261, EP 409, Ecuador: Azuay: Cuenca, 2650 m; LM 1053–54, KU 203457–58, Ecuador: Azuay: 3.7 km S Saraguro, 2800 m; LM 1055, KU 203443, Ecuador: Azuay: 7.1 km N San Lucas, 2940 m; LM 1091, KU 203444, Ecuador: Azuay: 3.7 km S Saraguro, 2800 m; LM 1093, KU 203470, Ecuador: Azuay: 2 km SSE Palmas, 2340 m; LM 1169, KU 203459, Ecuador: Azuay: 11.5 km SE Gualaceo, 2940 m; LM 1244–47, KU 178561–64, Ecuador: Bolívar: 5.4 km S Guranda, 2680 m; LM 1068–71, EP 461–64, Ecuador: Cañar: Hacienda Popoloma, near Cañar; LM 1094, KU 203474, Ecuador: Cañar: 3 km S Cañar, 3450 m; LM 1095, KU 203477, Ecuador: Cañar: Ingapirca, 3140 m; LM 1096, KU 203537, Ecuador: Cañar: 4 km N Zhud, 3040 m; LM 1238, KU 178553, Ecuador: Chimborazo: 3 km S Guamote, 3200 m; LM 1251–1256, KU 178547–52, Ecuador: Chimborazo: 10.5 km SSW Mocha, 3450 m; LM 1062–63, EP 486, Napo: Páramo de Antisana; LM 1240–43, EP 935, Ecuador: Pichincha: Machachi.

Gastrotheca psychrophila.—LM 375, KU 142634–37, Ecuador: Zamora-Chinchipe: 13.5 km E Loja, 2800 m. *G. riobambae.*—LM 1057–61, EP 481–85, Ecuador: Cotopaxi: Latacunga, 2750 m; LM 86, KU 178465–69, Ecuador: Imbabura: Lago Cuicocha, 3010 m; LM 1257, KU 178554, Ecuador: Pichincha: Paso de Guamaní, 3860 m; LM 74, UIMNH 94913–14, Ecuador: Pichincha: Quito, 2850 m; LM 1065–67, EP 424, 466–67, Ecuador: Pichincha: Sangolqui; LM 1170, KU 203527, Ecuador: Tungurahua: 1.1 km SW Pelileo, 2520 m. *G. ruizi.*—LM 1086, KU 20002, Colombia: Putumayo: Santiago, 2250 m. *G. trachyceps.*—LM 349, KU 181188–90, Colombia: Cauca: W slope Cerro Munchique, 29 km WNW El Tambo, 2530 m. *G. weinlandii.*—LM 377, KU 143105–06, Ecuador: Napo: 16.5 km NNE Santa Rosa, 1700 m.

Hemiphractus bubalus.—LM 95, KU 178588, Ecuador: Pastaza: Mera, 1100 m. *H. fasciatus.*—LM 1138, UIMNH 94542, Panama: Chiriquí: Continental divide above Quebrada La Arena, 1250–1400 m. *Stefania evansi.*—LM 78, KU 181112–13, Venezuela: Bolívar: La Escalera, km 112 on El Dorado-Santa Elena de Uairén road, 900 m. *S. ginesi.*—LM 1056, SG 1864–65, Venezuela: Amazonas: Abacapa-tepui.

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Copeia, 1988(3), pp. 543–554

Variation and Systematics of *Etheostoma radiosum*, the Orangebelly Darter (Pisces: Percidae)

WILLIAM J. MATTHEWS AND FRANCES P. GELWICK

Until recently, the known range of *Etheostoma radiosum* (orangebelly darter) extended from the Ouachita River of southwestern Arkansas westward to the Blue River system of southcentral Oklahoma. Since 1952, the *E. radiosum* complex has been recognized as consisting of three subspecies: *E. radiosum radiosum* (Ouachita and Little rivers, Arkansas and Oklahoma), *E. r. paludosum* (Kiamichi and Boggy rivers, Oklahoma), and *E. r. cyanorum* (Blue River). Our recent discovery of *E. radiosum* populations in creeks of the Washita River extended the known range of the species westward, and prompted reassessment of the recognized subspecies. On the basis of multivariate analyses of meristic and morphometric character suites, and reexamination of the univariate characters previously used to diagnose subspecies, we recommend that three subspecies of *E. radiosum* continue to be recognized, with the newly discovered populations in the Washita assigned to *E. r. paludosum*. This action is supported by well-defined characters that separate *E. r. cyanorum* from other forms of the complex, and by a unique shared character of coloration that unites forms in the Kiamichi-Boggy-Washita systems and separates them from the rest of the complex.

ETHEOSTOMA radiosum, the orangebelly darter, is a complex of forms endemic to southwest Arkansas and southeast Oklahoma, above the Fall Line (Retzer et al., 1986). Originally described as a subspecies of *Etheostoma whipplei* by Hubbs and Black (1941), *E. radiosum* was elevated to species rank by Moore and Rigney (1952), who recognized three subspecies: *E. radiosum radiosum*, of the Ouachita and Little River systems, Oklahoma and Arkansas; *E. r.*

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